Generation of an iPSC line from a patient with GTP cyclohydrolase 1 (GCH1) deficiency: HDMC0061i-GCH1

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Abstract: Fibroblasts from a female patient carrying a heterozygous variation in GTP cyclohydrolase 1 (GCH1; OMIM: 600225; HGNC: 4193; $c.235_240del/p.(L79_S80del)$), therate−limitingenzymeoftetrahydrobiopterin($BH4$ $iPS2.0SendaiReprogrammingKit(Invitrogen)deliveringthefourreprogrammingfactorsOct3/4, Sox2, c−MycandKlf4.PluripotencyofHDMC0061i−GCH1wasverifiedusingimmunohistochemistryandRT−PCRanalysis.Cellsdifferentiatedspontaneouslyintothe3germlayersinvitroandpresentedanormalkaryotype.HDMC00$ $GCH1representsthefirstmodelsystemtoelucidatethepathomechanismunderlyingthisraremetabolicdiseaseandausefult$

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Lab Resource: Stem Cell Line

Generation of an iPSC line from a patient with GTP cyclohydrolase 1 (*GCH1*) deficiency: HDMC0061i-GCH1

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ABSTRACT

Fibroblasts from a female patient carrying a heterozygous variation in GTP cyclohydrolase 1 (*GCH1*; OMIM: 600225; HGNC: 4193; c.235_240del/p.(L79_S80del)), the rate-limiting enzyme of tetrahydrobiopterin (BH_4) synthesis, were reprogrammed to iPSCs using the Cytotune®-iPS 2.0 Sendai Reprogramming Kit (Invitrogen) delivering the four reprogramming factors *Oct3/4*, *Sox2*, *c-Myc* and *Klf4*. Pluripotency of HDMC0061i-GCH1 was verified using immunohistochemistry and RT-PCR analysis. Cells differentiated spontaneously into the 3 germ layers in vitro and presented a normal karyotype. HDMC0061i-GCH1 represents the first model system to elucidate the pathomechanism underlying this rare metabolic disease and a useful tool for drug testing.

Resource table

Name of stem cell line	HDMC0061i-GCH1
Institution	Department of General Pediatrics, Division of Neuropediatrics and Metabolic Medicine, University Hospital Heidelberg, Heidelberg, Germany
Person who created resource	Sabine Jung-Klawitter
Contact person and email	Sabine Jung-Klawitter; Sabine.Jung-Klawitter@med.uni-heidelberg.de
Date archived/stock date	August 2016
Origin	human fibroblasts
Type of resource	Human induced pluripotent stem cell (iPSC); generated from a female patient suffering from a heterozygous variation in the *GCH1* gene (NC_000014.9; OMIM: 600225; Gene ID:2643; c.235_240del/p.(L79_S80del)); manually picked single clone
Sub-type	Induced Pluripotent Stem Cells (iPSCs)
Key transcription factors	hOct3/4, hSox2, hc-Myc, hKlf4 (Cytotune®-iPS 2.0 Sendai Reprogramming Kit – Invitrogen, Thermo Fisher Scientific Inc.)
Authentication	Identity and purity of cell line confirmed as shown in Fig. 1
Link to related literature	López-Laso et al. (2012); Dyskinesias as a limiting factor in the treatment of Segawa disease. Pediatric Neurology 46:404–406.
Information in public databases	OMIM: 600225; HGNC ID: HGNC: 4193; Gene ID:2643
Ethics	Institutional ethics committee approval obtained (No. 2016-02-04 ZB 52315)/Patient written informed consent obtained

1. Resource details

Fibroblasts from a female patient carrying a heterozygous variation in GTP cyclohydrolase 1 (*GCH1*; OMIM: 600225; c.235_240del/p.(L79_S80del); López-Laso et al., 2012) were reprogrammed using the Cytotune®-iPS 2.0 Sendai Reprogramming Kit (Invitrogen) delivering the four human reprogramming factors Oct3/4, Sox2, c-Myc and Klf4. Fluorescence R-banding was performed and provided a normal diploid 46, XX karyotype (Fig. 1A). RT-PCR with Sendai virus-specific primers (Table 1) was used to show absence of Sendai virus vector (SeV; Fig. 1B). Absence of mycoplasma contamination in the parental fibroblasts, during reprogramming and in the iPSC line itself was verified by using a PCR Mycoplasma Test Kit (AppliChem) (Fig. 1C). Presence of the heterozygous variant was verified by PCR and subsequent Sanger Sequencing (Fig. 1D). Pluripotency marker gene expression was analyzed by RT-PCR with gene-specific primers (Fig. 1E; Table 1) as well as via immunofluorescence staining for the pluripotency markers Oct3/4, Sox2, Nanog, Lin28 and SSEA-4 (Fig. 1F). Embryoid body (EB) formation followed by immunofluorescence staining showing the presence of endodermal (AFP), mesodermal (SMA) and ectodermal (βIII-tubulin) markers (Fig. 1G) and RT-PCR amplification of AFP (endoderm), TBX20 (mesoderm) and Nestin (ectoderm; Fig. 1H; Haase et al., 2009) was used to show the differentiation potential of the iPSCs.

2. Materials and methods

2.1. iPSC reprogramming

Fibroblasts were cultured in DMEM (Invitrogen) supplemented with 10% FBS (Invitrogen), 1% Penicillin/Streptomycin (Invitrogen), 0.1 mM

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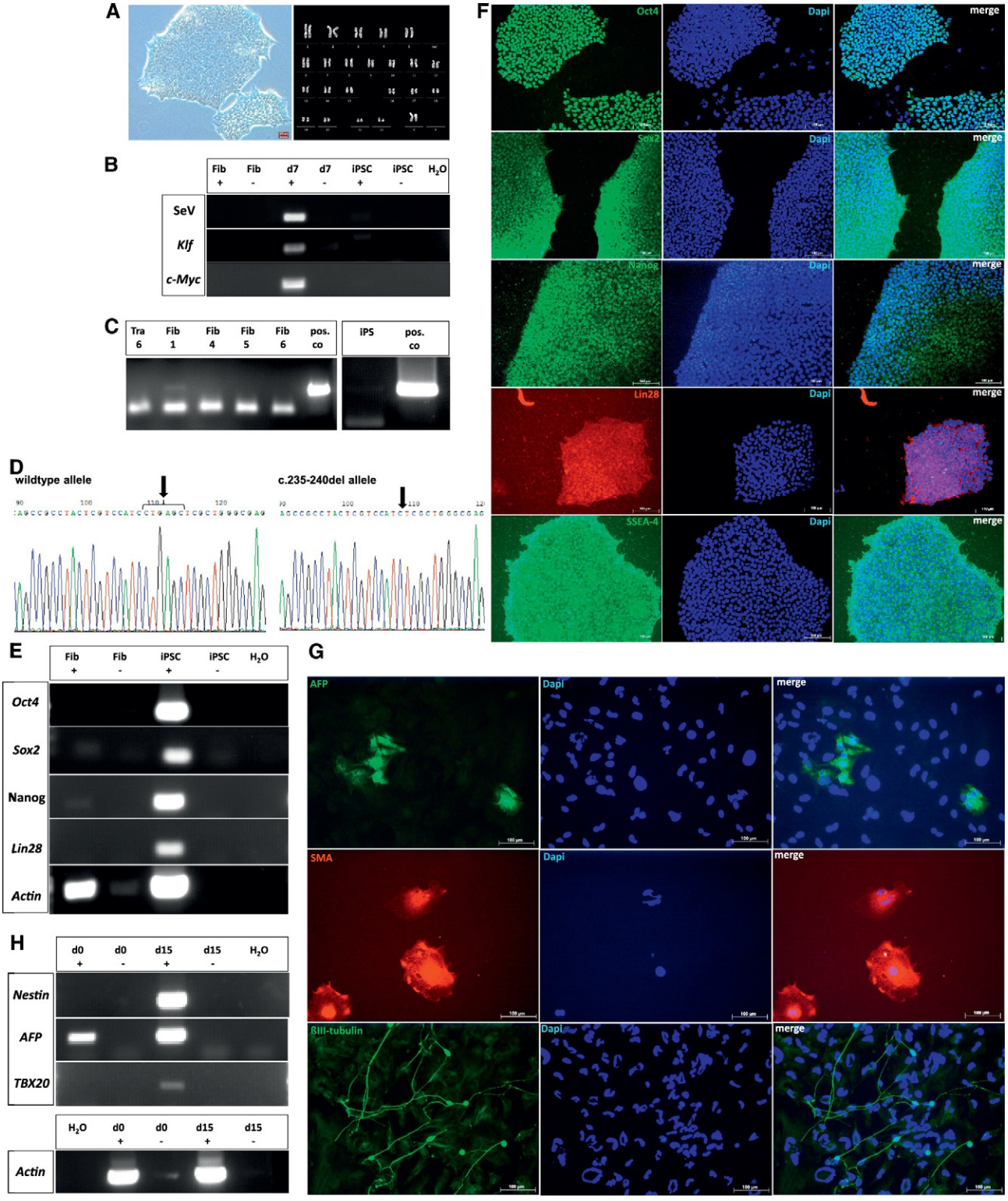

Fig. 1. HDMC0061i-GCH1 iPSCs (p5) display a normal diploid karyotype (46, XX; A). Sendai virus was not detectable in untransduced fibroblasts of the patient (Fib) or in iPSCs (iPSC, p7) but in fibroblasts seven days after transduction (d7; B). No mycoplasma contamination was present in the fibroblasts (Fib 6), during reprogramming (Tra6) or in the iPSCs itself (iPS) (C). Presence of the heterozygous 6 bp deletion was verified by Sanger Sequencing showing presence of both the wildtype allele (marked as wildtype allele) and the deletion (marked as c.235_240del allele) in the same DNA preparation of HDMC0061i-GCH1 (D). HDMC0061i-GCH1 iPSCs express several pluripotency marker genes as shown by RT-PCR (E) as well as by immunofluorescence staining (F). HDMC0061i-GCH1 iPSCs can be differentiated into all three germ layers as shown by immunofluorescence staining (G) and RT-PCR (H). Abbreviations: +: cDNA synthesis with reverse transcriptase; −: cDNA synthesis without reverse transcriptase; Tra6: Patient-specific fibroblasts during reprogramming; Fib1, Fib4, Fib5, Fib6: fibroblasts isolated from different patients whereby Fib6 were used to generate HDMC0061i-GCH; iPS: HDMC0061i-GCH iPSCs; pos. co: positive control to show functionality of mycoplasma detection in the PCR; d0: undifferentiated iPSCs; d15: differentiated iPSCs on day 15 of differentiation; d7: patient-specific fibroblasts on d7 after transduction with Sendai virus. Scale bars represent 100 μm.

Table 1
Primers used in the study.

Gene symbol	Sequence (fwd and rvs; 5′–3′)	Product size (bp)	Annealing temperature (°C)
β-actin	CATGGAGAAAATCTGGCACCAC GCACAGCTTCTCCTTAATGTCAC	409	56
Oct3/4	GAACCAGTATCGAGAACCG TCAGTTTGAATGCATGGGAG	383	56
Sox2	CACATGTCCCAGCACTACCAG CACATGTGTGAGAGGGGCAG	77	56
Nanog	AAACAGAAGACCAGAACTGTG CAGTTGTTTTTCTGCCACCTCT	191	56
Lin28	CCATATGGTAGCCTCATGTC CAATTCTGTGCCTCCGGG	126	56
Nestin	CAGCGTTGGAACAGAGGTTGG TGGCACAGGTGTCTCAAGGGTAG	388	62
AFP	ACTCCAGTAAACCCTGGTGTTG GAAATCTGCAATGACAGCCTCA	255	55
TBX20	AGGTACCGCTACGCCTAC GTCAGTGAGCCTGGAGGA	470	50
SeV (Sendai)	GGATCACTAGGTGATATCGAGC ACCAGACAAGAGTTTAAGAGATATGTATC	181	55
Klf4 (Sendai)	TTCCTGCATGCCAGAGGAGCCC AATGTATCGAAGGTGCTCAA	410	55
c-Myc (Sendai)	TAACTGACTAGCAGGCTTGTCG TCCACATACAGTCCTGGATGATGATG	532	55
GCH1	CAGCGAGGAGGATAACG CGTTTAGGACATCTGAGATG	179	55

non-essential amino acids (Invitrogen) and 50 μM β-mercaptoethanol (Invitrogen) and reprogrammed in passage 7 applying the Cytotune®-iPS 2.0 Sendai Reprogramming Kit (Invitrogen) following the manufacturer's instructions. Briefly, cells were seeded at a density of 1×10^5 cells/well the day before transduction into two wells of a Matrigel (BD Biosciences)-coated 6 well plate (Greiner). On the day of transduction, one well was counted and necessary volumes for transduction of each virus were calculated. Cells were transduced with an MOI of 5 for KOS virus and hc-Myc virus, respectively, and an MOI of 3 for hKlf4 virus in a total volume of 1 ml growth medium. The following day, medium was changed and cells were cultivated for 6 additional days with a daily medium change. On day 7, transduced fibroblasts were trypsinized, counted, and seeded on 1×10^6 Mitomycin-treated murine embryonic fibroblasts (MEF; Merck-Millipore) per 100 cm gelatine-coated (0,1% gelatine (Sigma) in MilliQ water) petri dish (Sarstedt) at a density of 2×10^5 transduced cells/plate, 1×10^5 transduced cells/plate and 1×10^4 transduced cells/plate, respectively. Remaining cells were washed with D-PBS (Invitrogen), spun down, frozen and used as positive control for SeV-specific RT-PCR. The following day, medium was changed to ESC medium (KnockOut DMEM, 20% Serum Replacement, 0.1 mM non-essential amino acids, 50 μM β-mercaptoethanol, 1% penicillin/streptomycin (all from Invitrogen) and 4 ng/ml bFGF (Peprotech)) and cells were cultivated for 21–27 more days with daily medium change. Between days 21 and 27 post transduction colonies with iPS-like morphology were manually picked, transferred to Matrigel-coated (BD Biosciences) 12well plates (Greiner) containing Essential 8™ Flex Medium (Invitrogen) and further expanded in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.2. RT-PCR analysis

RNA was extracted with Trizol® (Invitrogen) according to the manufacturer's instructions. 1 μg RNA was digested with DNaseI (Invitrogen) to remove DNA contaminations and cDNA was synthesized from 0.5 μg DNaseI-digested RNA using SuperScript® III Reverse Transcriptase and Oligo $(dT)_{20}$ primer (both from Invitrogen) according to the manufacturer's instructions. Expression of endogenous pluripotency markers (*Oct3/4*, *Sox2*, *Nanog*, *Lin28*) or germ layer markers (*Nestin*, *AFP*, *TBX20*) was monitored using Crimson *Taq* Polymerase (New England Biolabs) and standard PCR conditions (95 °C 30 s (1×); 95 °C 30 s, 55–62 °C 1 min, 68 °C 1 min (35×); 68 °C 5 min (1×), 12 °C (hold); for details see Table 1). To show absence of Sendai virus, GoTaq® Green Mastermix (Promega) was used for PCR (94 °C 2 min (1×); 94 °C 30 s, 55 °C 30 s, 72 °C 30 s (35×); 72 °C 5 min (1×), 12 °C (hold)). PCR products were analyzed using agarose gelelectrophoresis.

2.3. In vitro differentiation via embryoid body formation

iPSCs were harvested with ReLeSR™ (Stem Cell Technologies), singled and counted. 9×10^5cells/well were transferred to an AggreWell 800™ plate (Stem Cell Technologies) which contained ESC medium supplemented with 10 μM ROCK inhibitor (Y-27632; SIGMA) but without bFGF to form uniform embryoid bodies (EBs) overnight. The next day, EBs were transferred to an Ultra-low attachment plate (Greiner) and cultivated for 6 additional days in ESC medium without bFGF. On day 8, EBs were trypsinized and seeded onto gelatine coated plates (containing cover slips for immunofluorescence staining or nothing for RNA isolation and RT-PCR). Cells were then cultured in differentiation medium (ESC medium without bFGF/MEF medium 50:50 (v/v)) for 7 more days. On day 15, cells were fixed with 4% paraformaldehyde (PFA; Sigma) in PBS for immunofluorescence staining or lysed using Trizol® for RNA isolation and RT-PCR.

2.4. Immunofluorescence staining

Presence of the pluripotency marker genes Oct3/4, Sox2, Nanog, Lin28, and SSEA-4 or germ layer markers (AFP, ßIII tubulin, SMA) was analyzed via immunofluorescence staining. For detection of pluripotency marker genes, iPSCs were seeded onto Matrigel-coated (BD Biosciences) cover slips and cultivated in Essential 8™ Flex medium (Invitrogen) for 3–5 days before fixation. iPSCs or differentiated cells after EB formation were fixed in 4% PFA/PBS (15 min, room temperature (RT)) and permeabilized with 1% Triton X-100/PBS for 10 min at RT. Blocking was performed with 5% BSA/0.1% Triton X-100/PBS for one hour at RT. Then, cells were incubated in 5% BSA/PBS with primary antibodies (Table 2) for one hour at RT, followed by an incubation step with the secondary antibodies (Table 2) in 5% BSA/PBS for 30 min at RT. Cells were mounted in Fluoromount-G with DAPI (eBioscience) to counterstain the nuclei and analyzed using a fluorescence microscope (Leica) and Leica Imaging Software.

2.5. Karyotyping

For Karyotyping, cells were trypsinized and metaphases were prepared according to standard procedures. Fluorescence R-banding using chromomycin A3 and methyl green was performed as described

Table 2
Antibodies used for immunofluorescence staining.

Application	Antibody	Dilution	Company	Cat. #
Pluripotency	Mouse anti-Oct4	1:100	SantaCruz	sc-5279
	Mouse anti-Sox2	1:100	Merck Millipore	MAB4343
	Mouse anti-Nanog	1:100	Thermo Fisher	MA1–017
	Mouse anti-SSEA4	1:100	SantaCruz	sc-21704
	Rabbit anti-Lin28	1:100	SantaCruz	sc-67266
	Mouse anti-AFP	1:100	Abcam	ab3980
Differentiation	Rabbit anti-SMA	1:100	Abcam	ab5694
	Mouse anti-ßIII-Tubulin	1:100	Abcam	ab78078
Secondary antibodies	Alexa Fluor goat anti mouse 488	1:500	Thermo Fisher	A-10667
	Goat anti-Rabbit IgG (H + L), TRITC conjugate	1:500	Thermo Fisher	A16101

in detail earlier (Schlegelberger et al., 1999). At least 20 metaphase spreads were analyzed and evaluation was carried out at a minimum level of 300 bands. Chromosomes were classified according to the International System for Human Cytogenetic Nomenclature (ISCN 2013; Shaffer et al., 2013).

2.5. DNA isolation and sanger sequencing

To confirm the presence of the heterozygous variation in the *GCH1* gene, specific primers (Table 1) were designed for the amplification of the genomic region of interest. DNA was extracted from the iPSCs using DNAzol® (MRC Inc.) according to the manufacturer's instructions. 100 ng DNA was used for PCR with Crimson *Taq* Polymerase (New England Biolabs) using standard PCR conditions (95 °C 30 s (1×); 95 °C 30 s, 55 °C 30 s, 68 °C 30 s (35×); 68 °C 5 min (1×), 12 °C (hold); for details see Table 1). PCR products were cloned into pGEM®-T Easy (Promega) and 10 clones where sequenced by GATC Biotech (Köln, Germany).

2.6. Detection of mycoplasma contamination

To show that the parental fibroblasts and the derived iPSCs are free of mycoplasma contamination the PCR Mycoplasma Test Kit (AppliChem) was used according to the manufacturer's instructions. In brief, 1.0 ml of cell culture supernatant was transferred into a 2.0 ml centrifuge tube. Cellular debris was pelleted by centrifugation at 250 ×*g* for 3 min. The supernatant was transferred to a new 1.5 ml tube, and centrifuged for 10 min at 13.000 rpm at room temperature. The supernatant was discarded, the pellet was re-suspended in 50 μl Buffer Solution, and heated for 3 min at 95 °C. Amplification of the 16srRNA of a potential Mycoplasma contamination was performed with the Reaction Mix provided by the manufacturer in a volume of 25 μl using the following PCR conditions: 94 °C 30 s (1×); 94 °C 30 s, 60 °C 2 min, 72 °C 1 min (35×); 94 °C 30 s, 60 °C 2 min, 72 °C 5 min (1×), 12° (hold) followed by agarose gel electrophoresis.

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